Killing of Blood-Stage Murine Malaria Parasites by Hydrogen Peroxide

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Both nonlethal *Plasmodium yoelii* and lethal *Plasmodium berghei* were killed in vitro by hydrogen peroxide at concentrations as low as 10^{-5} M. Higher concentrations were required in the presence of added normal erythrocytes. Injection of hydrogen peroxide in vivo significantly reduced *P. yoelii* parasitemia but had less effect on *P. berghei*.

The process by which immune animals destroy malaria parasites is still not fully understood. Passive transfer of monoclonal antibodies (4) has confirmed the importance of blocking penetration of merozoites into the erythrocyte (2), but experiments in agammaglobulinemic mice have demonstrated the existence of non-antibody-dependent mechanisms apparently involving specific T cells (5), whereas nonspecific macrophage activation can also confer protection in certain host-parasite combinations (1). We have shown elsewhere that serum containing "tumor necrosis factor," a product of activated macrophages (8), can kill blood-stage malaria parasites in vitro (15) and in vivo (14). We now present evidence that hydrogen peroxide, which can also be released by macrophages, is effective against murine blood-stage malaria at concentrations which might occur naturally.

We used four rodent parasites chosen for their various degrees of virulence: *Plasmodium chabaudi* and *Plasmodium yoelii* 17X, which give self-resolving infections in mice; a stable lethal variant of *P. yoelii* 17X, which kills mice within 14 days but against which vaccination is protective (13); and *Plasmodium berghei* (ANKA), fatal to mice within 18 days and relatively resistant to vaccination. Infections were established with 10^4 parasitized erythrocytes injected intravenously, and blood parasitemia was followed by determining the percentage of parasitized erythrocytes in Giemsa-stained tail blood smears.

Killing of parasites in vitro was measured by using an "infectivity assay" similar to that with which we have previously demonstrated the effect of mononuclear cells (16) and of "tumor necrosis serum" (15). Briefly, parasitized blood containing approximately 10^4 parasitized erythrocytes and 5×10^5 uninfected erythrocytes was incubated in 1-ml volumes of *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES)-

buffered RPMI 1640 containing 10% fetal calf serum in 24-well Linbro plates. Additional washed normal mouse erythrocytes were added to some cultures to give final erythrocyte concentrations of 5×10^6, 5×10^7, and 5×10^8 per ml. Appropriate dilutions of hydrogen peroxide (20 volumes; 6% [wt/vol]; Hopkin & Williams) or distilled water (control) were added to give final peroxide concentrations ranging from 10^{-6} to 4×10^{-2} M. After incubation for 1 h at 37°C in an atmosphere of 5% CO_2 in air, samples of 0.2 ml were injected intravenously in triplicate into outbred Tuck no. 1 female mice, and their parasitemia was counted daily. The log dose of viable parasites injected was determined from the time taken to reach a parasitemia of 0.5% by reference to a standard curve. There is an inverse relationship between the time taken to reach a given parasitemia and the log dose of viable parasites injected (15). When hydrogen peroxide was added to the cultures at concentrations of from 10^{-2} to 10^{-4} M, all the parasites were killed, and no parasites grew in the recipient mice. With the nonlethal *P. yoelii*, the addition of various numbers of normal erythrocytes improved parasite survival and reduced the effect of hydrogen peroxide; nevertheless, even in the presence of 5×10^8 added erythrocytes, there was detectable killing with 10^{-2} M peroxide. *P. berghei* appeared to be equally or more susceptible (Fig. 1). *P. chabaudi* and the lethal *P. yoelii* do not survive as well in vitro under these conditions and were not included in this experiment.

Hydrogen peroxide was also effective against parasites in vivo. Intravenous injection of 1 mg, the highest dose which caused the mice no visible distress, on day 5 or 6 of infection resulted in a significant reduction of parasitemia 2 h later, and a second injection led to a further fall. CBA and (BALB/c × C57Bl) F1 mice gave identical results, but as Fig. 2 shows, not all the
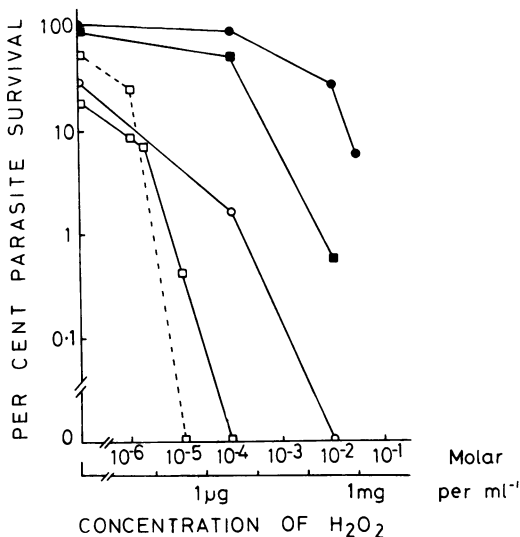
FIG. 1. Mean parasite survival after incubation in various concentrations of hydrogen peroxide, expressed as a percentage of the number of viable parasites added at time zero. Solid lines, nonlethal *P. yoelii* with 5×10^8 (●), 5×10^7 (■), 5×10^6 (○), or no added normal erythrocytes (□). Broken line, *P. berghei* without added erythrocytes. Each point represents two to four separate experiments.

parasites were equally susceptible, *P. chabaudi* and the lethal *P. yoelii* being the most and *P. berghei* the least affected. When the parasitemias of individual mice were compared from 0 to 4 h by using a paired *t* test, the hydrogen peroxide-treated groups showed a significant fall for lethal *P. yoelii* ($P < 0.005$), *P. chabaudi* ($P < 0.01$ to 0.005), and nonlethal *P. yoelii* ($P < 0.01$ to 0.05); for *P. berghei*, the decrease was not significant. In all experiments, the control groups showed either no significant change or a slight increase. When the mean parasitemias of hydrogen peroxide and control groups were compared by *t* test at 4 h, the differences were significant for *P. chabaudi* and both lethal and nonlethal *P. yoelii* ($P < 0.005$). At this stage of the infection, *P. berghei* is restricted to the reticulocytes and the other two species to the adult erythrocytes; thus, the maturity of the host erythrocyte could be a major influence on susceptibility to hydrogen peroxide. However, the nonlethal *P. yoelii*, also restricted to reticulocytes, appeared to be relatively susceptible; thus, another possibility is that parasite species differ in their production of catalase or other antioxidants, such as have been found in the intracellular protozoan *Toxoplasma gondii* (9) and in mycobacteria differing in their susceptibility to hydrogen peroxide (6). It remains unclear why the species variation was so marked in

vivo but not in vitro, but the same effect has been noted with tumor necrosis serum (14); perhaps there are relatively more *P. berghei* in tissue sites to which these toxic molecules do not penetrate. It is possible that the hydrogen peroxide acts by making the microenvironment of the erythrocyte inhospitable for the parasite (i.e., by altering the hemoglobin), but it does not seem to have a detectable effect on erythrocyte numbers as measured by hematocrit levels (unpublished data). Moreover, any effect on nonparasitized erythrocytes would be expected to affect all four parasites equally. In one experiment with repeated injections of hydrogen peroxide, it was possible to keep a *P. chabaudi* parasitemia significantly below control values for at least 3 days, and in another, three of five mice given hydrogen peroxide on day 5 eventually recovered from the uniformly lethal *P. yoelii*. However, we have not been able to induce rapid elimination of infection. Of course, it is likely that even this relatively large amount of hydrogen peroxide is rapidly inactivated by endogenous catalase (e.g., from erythrocytes [Fig. 1]) and that only a proportion of the parasites in the blood are exposed to toxic levels.

Hydrogen peroxide released from macrophages or neutrophils in vivo would be expected to be most effective in sites at which the parasite and the cells come into close contact, such as the sinusoids of the liver or the spleen. Liver macrophages are activated during malaria infections, as measured by secretion of plasminogen activator (H. M. Dockrell and J. H. L. Playfair, submitted for publication), and can respond to infection with *Leishmania* by giving an oxidative burst (J. Y. Channon and J. M. Blackwell, personal communication). Moreover, peritoneal cells induced with fetal calf serum during a malaria infection show spontaneous secretion of hydrogen peroxide (unpublished results). Liver macrophages, as well as those in other sites, extensively phagocytose parasitized erythrocytes, as seen by intracellular pigment (3); thus, it is possible that this acts as a trigger for an oxidative burst, similar to agents like phorbol myristate acetate used in vitro.

An effect of parenteral hydrogen peroxide has also been shown in a tumor system; 8 mg administered intraperitoneally was able to kill more than 90% of P338 lymphoma cells (11). It has been calculated that 10^6 activated macrophages can release about 2 nmol of hydrogen peroxide per min (12), so that, at a rough estimate, the macrophages in the mouse liver during malaria, which number at least 10^8 (3), could release some 200 nmol (6 μg) of hydrogen peroxide per min. Taking into account that in sites such as the microenvironment of the spleen or the liver, the inhibition by erythrocyte catalase
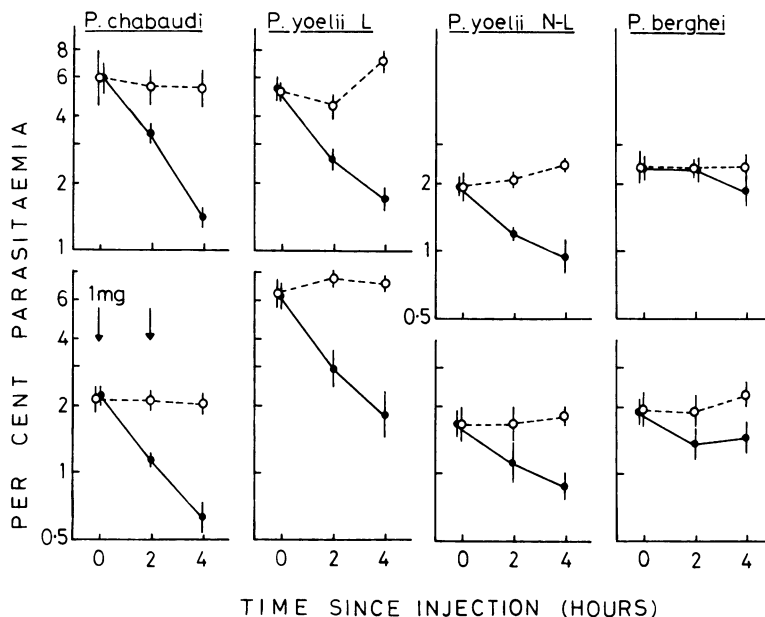
TIME SINCE INJECTION (HOURS)

FIG. 2. CBA or (BALB/c × C57Bl) F1 mice were injected intravenously with 10^4 of the parasites indicated. Five days later, they were injected intravenously twice with either 1 mg of hydrogen peroxide (0.25 ml of a 1/16 dilution of 20 volumes of hydrogen peroxide [6% (wt/vol); Hopkin & Williams] [solid lines]) or with 0.25 ml of phosphate-buffered saline (broken lines). Injections were at 0 and 2 h, as indicated by arrows in the lower left panel, and the percentage of infected erythrocytes was counted at 0, 2, and 4 h on Giemsa-stained tail blood smears. The values plotted are the geometric means ± 1 standard error of groups of four to nine mice. Two separate experiments are shown for each species of parasite. With the exception of *P. berghei*, all the hydrogen peroxide groups showed a significant drop in parasitemia from 0 to 4 h, whereas the 4-h values are significantly below those of the control groups ($P < 0.005$) when assessed by Student's t test.

might be relatively less efficient than in the bloodstream, it is evident that the concentrations shown here to be toxic (less than 1 μg/ml in vitro [Fig. 1]) are not beyond attainment in vivo, and we propose that hydrogen peroxide is in fact a possible contributor to the destruction of at least some species of malaria parasite. From the point of view of vaccination against this disease, it is encouraging that activation of macrophages, for example, by specific T cells, can increase their output of hydrogen peroxide (7), whereas the presence of specific antibody has been shown to facilitate the lysis of target cells by hydrogen peroxide (10). We are currently investigating the susceptibility of both human and mouse malaria parasites to in vitro systems generating hydrogen peroxide and other intermediates during the oxidative burst, as well as the production of such factors by resident tissue macrophages during murine malaria infections.

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